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HBV Core Promoter Mutations Prevail in Patients With Hepatocellular Carcinoma From Guangxi, China

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The development of primary liver cancer frequently is associated with persistent HBV infection, and tumours may arise in individuals who are anti-HBe positive. However, it is unlear whether viruses with an HBeAg-negative phenotype are associated with tumour development or are selected, during seroconversion, after chromosomal integration of wild-type viral DNA. In order to investigate the temporal evolution of the HBV genome in such individuals, the polymerase chain reaction was used to amplify HBV DNA from tumour tissue and serum of 14 patients from Guangxi, China with hepatocellular carcinoma. Comparison of nucleotide and amino acid sequences of the precore and proximal core region of HBV from the two sites in each patient produced evidence of divergence following integration in the tumour, but in most cases, HBeAg-negativity could not be explained by precore mutations. Sequences from the core promoter region were therefore examined and mutations were found in the majority, which are believed to upregulate transcription of the core (and pregenomic) RNA but to downregulate precore mRNA. To determine whether this finding merely reflected sequence variation among geographical isolates of HBV, the same region of HBV DNA from asymptomatic controls was sequenced and these mutations were found to be rare. We hypothesise that HBV with the core promoter mutations replicates at higher levels than the wild type, with the consequence that more integrations occur into the hepatocyte chromosomes during the early stages of infection. These hepatocytes may expand clonally and be targets for further mutagenic events leading to tumour development. ***J. Med. Virol. 56:18–24, 1998.***

KEY WORDS: antigen negative; precore mutants; integration; PCR; sequence analysis

INTRODUCTION

The causal role of persistent hepatitis B virus (HBV) infection in the development of hepatocellular carcinoma (HCC) is well established [Robinson, 1989]. The association is particularly strong in regions of the world where HBV infection is highly prevalent, such as parts of China, southeast Asia and sub-Saharan Africa. The incidence of HCC in Guangxi province, southern China, is among the highest in the world and >90% of cases are in individuals positive for hepatitis B surface antigen (HBsAg) in serum [Yeh et al., 1989]. The prevalence of antibody to hepatitis C virus is low in Guangxi, but contamination of foodstuffs with aflatoxin is common [Yeh et al., 1989; Okuno et al., 1994; Yuan et al., 1996].

Development of HCC in HBsAg-positive individuals frequently follows a prolonged period of virus replication. During this time, subgenomic fragments of HBV DNA may integrate into random sites in the hepatocyte chromosomes. Detection of integrated HBV DNA by Southern hybridisation of liver biopsies from persistently infected individuals without tumours [Shafritz et al., 1981; Harrison et al., 1986] implies clonal expansion following the initial integration event. Such expanded clones may be subject to selection (e.g., by the immune response to viral gene products that may be expressed) and further mutagenic events. These may be mediated by agents such as aflatoxin and its me-

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tabolites and affect genes involved in oncogenesis such as the p53 tumour suppressor gene [Ozturk, 1991].

HBsAg-positive patients with HCC often are negative for HBeAg, a marker of replication of wild-type virus. Such patients may remain viraemic, with HBeAg-negative variants, or have cleared replicating virus since the initial integration event(s). In the former case, evolution and immune selection of HBeAg-negative variants may have occurred following integration into pre-neoplastic cells. Alternatively, replication and integration of HBeAg-negative variants may be a risk factor for development of HCC [Hadziyannis et al., 1991]. Investigation of HBV DNA sequences from paired tumour and serum samples may provide information on the temporal evolution of viral sequences in the individual. Integrated HBV DNA is replicated along with chromosomal DNA in the tumour, with an extremely low rate of error, so that the sequence will remain unchanged over decades. In contrast, the sequences of circulating viral genomes are the result of error-prone replication and selection by factors such as fitness and the host immune response, and form a quasispecies that evolves over time.

We investigated HBV DNA sequences from 14 patients from Guangxi and for whom paired serum and tumour tissue were available, following amplification of the precore and part of the core region using the polymerase chain reaction (PCR). Since HBeAg negativity in the majority of patients could not be explained by precore mutations, core promoter sequences were also investigated. In addition, we investigated HBV DNA from 11 HBsAg-positive, asymptomatic individuals from the same geographical region to determine whether the high frequency of core promoter mutations found in the HCC patients merely reflected the predominant strains of HBV.

MATERIALS AND METHODS

Patients

Paired serum and tumour tissue were available from 16 patients (10 males and 6 females) diagnosed in Guangxi, Southern China, with HCC. Serum samples were tested for HBV DNA using nested PCR with primers from precore/core region. Two were negative and were excluded from the study group for sequence analysis; further details of the remainder are given in Table I. All except one (C15) were HBsAg positive.

TABLE I. HBeAg/Anti-HBe Status of 14 Viraemic Patients and Results of PCR of HCC DNA

Patient no.	Gender/ age	HBeAg/ anti-HBe	Precore PCR	Core promoter PCR
C2	M60	–/+	+	+
C3	F38	–/+	+	–
C4	M38	–/+	+	+
C5	F46	–/+	+	+
C7	F34	–/+	+	+
C8	M54	–/+	+	+
C10	F45	–/+	+	–
C11	M50	–/+	+	–
C12	M37	–/+	+	+
C13	M31	–/+	+	+
C14	F40	–/+	+	+
C15[a]	F41	–/–	+	+
C23	M33	+/–	–	–
C24	M42	–/–	+	+

[a]Patient C15 tested negative for HBsAg in serum. Amplification and sequence analysis of the region of the surface open reading frame encoding the a determinant [11] revealed a point mutation that resulted in an alanine, rather than a glycine, codon at position 145.

Asymptomatic Controls

Serum samples were obtained from 20 HBsAg-positive patients attending an accident and emergency clinic in Nanning, Guangxi, for reasons unconnected with liver disease and who were not transfused.

Preparation of DNA Samples for the PCR

Serum. Ten μl of each serum sample was boiled with 90 μl PBS for 15 min and spun for 1 min in a microfuge; 20 μl of the supernatant was amplified in a 100 μl PCR reaction. Alternatively, samples were digested overnight with pronase (1 mg/ml), extracted with phenol chloroform and precipitated with ethanol.

Tumour. DNA was prepared from 1 cubic centimeter of tissue as described previously [Harrison et al., 1986]. Briefly, the tissue was pulverized in liquid nitrogen, lysed with 0.5% SDS, and digested overnight with 1 mg/ml pronase. The nucleic acid solution was extracted twice with phenol/chloroform and precipitated with ethanol. After digestion with RNase A and further extraction, the DNA was redissolved in a final volume of 0.5 ml TE (pH 8.0). Five μl of this solution was used for first round PCR.

PCR Amplification

To amplify the precore and proximal core region, the PCR was carried out as described previously [Valliammai et al., 1995] using primers HDM3 and MDC1 for the first round of amplification; 5 μl of first round PCR product was amplified in a 100 μl reaction using nested primers MDN5 and HDB2. For the core promoter region, the PCR was carried out using primers B935 (5′-GCGCTGCAGAACCTTTGTGGCTCCTCTG-3′) and HDB2 for first round amplification (30 cycles, 94°C for 1.5′, 50°C for 1.5′, 72°C for 5′ followed by a 10′ extension at 72°C, using a 100 μl reaction). Then, 5 μl of the product was amplified further in a 100 μl reaction with primers XSEQ1 and B936 (5′-GCGCTGCAGGCTTGAAACAGTGGGACTAT-3′, 30 cycles, 94°C for 1.5′, 50°C for 1.5′, 72°C for 2.5′ followed by a 10′ extension at 72°C). HBV DNA from control subjects was amplified using primers B935 and MDC1 for the first round, and 5 μl of the product was amplified further with primers XSEQ1 (5′-CCCCGTCTGTGCCTTCTCATCTG-3′) and HDB2. (The cycle parameters were the same as described above for the core promoter region in each case.)

Sequence Analysis of PCR Products

PCR products were cloned into the vector pCR2.1 (Invitrogen, Leek, The Netherlands) and mini preparations of DNA made from 1.5 ml of 5 ml cultures of individual colonies by phenol extraction and ethanol precipitation of the cell pellet. Plasmids with inserts were identified by digestion with EcoRI and the remainder of the cultures used for extraction of DNA using a QIAprep Spin kit (Qiagen, Crawley, UK). The purified DNA was denatured in the presence of 10 pmol primer using NaOH (1 μmole), neutralised with HCl (1 μmole), corrected to 1× sequenase buffer plus an additional 90 mM Tris-HCl pH7.5 and sequenced using Sequenase version 2.0 (Amersham International plc, Buckinghamshire, UK). The −40 and reverse sequencing primers were used along with HDB2 to sequence the precore region. Primers B936, XSEQ1 and XSEQ3 (5′-CATAAGAGGACTCTTGGACT-3′) were used to sequence the core promoter region.

RESULTS

Analysis of Precore/Proximal Core Region From HCC Patients

Sequence analysis was carried out on two to seven independent clones of the PCR products from the 14 patients' sera (Figs. 1 and 2). Although all but one patient were HBeAg-negative (Table I), this could be accounted for by precore codon 28 nonsense mutations in only three (C4, C5 and C12), with mixed sequences in two others (C8 and C13). Several studies have noted that the emergence of the 1896 mutation, which generates the codon 28 termination signal, may be constrained when it results in disruption of base pairing in the ϵ packaging signal [Li et al., 1993; Lok et al., 1994]. In circulating HBV from six of our patients (C2, C3, C10, C11, C15, and C23) the base at the relevant position was C rather that T, precluding development of the precore stop mutation.

The same methodology was used to amplify and sequence HBV DNA from tumour tissue. Amplicons were obtained from all tumours except that of patient C23 (Figs. 1 and 2). Notably, this is the only patient who was HBeAg positive. In order to confirm integration of HBV DNA in his tumour, sequences from the surface region were amplified [Harrison et al., 1991], that found most commonly in HBV integrants [Chen et al., 1988].

Comparison of the nucleotide sequences amplified from serum and tumour (Fig. 2) shows a significant difference between the two tissues. This probably reflects the accumulation of point mutations during HBV DNA replication, and nucleotide sequences from the tumours are less heterogeneous than those from serum. These differences in nucleotide sequence are reflected at the amino acid level in most patients (Fig.1). Variation in tumour sequences may be attributable to PCR amplification of more than one integrant. PCR of serum and tumour HBV DNA from patient C24 resulted in smaller amplimers than predicted and sequence analysis revealed a 63 nt, in frame, deletion leading to loss of 21 amino acids from the nucleocapsid protein (Fig. 1).

Analysis of Core Promoter Region From HCC Patients

The lack of circulating HBeAg could not be accounted for by the precore sequences from 10 of the 13 HBeAg-negative patients. Mutations in the region of the core promoter, which determines the cap site of the precore mRNA, may downregulate synthesis of HBeAg through a reduction of transcription of the precore mRNA and have been described frequently in HBV from HBeAg-negative Oriental patients [Okamoto et al., 1994; Takahashi et al., 1995]. We amplified and sequenced that region of HBV DNA from serum and tumour, and partial sequences are shown in Figure 3.

The core promoter region could not be amplified from tumour DNA from four patients (C3, C10, C11, and C23). This included the HBeAg-positive patient whose tumour was negative with precore region, but positive with surface region, primers. Integrants may be amplified using the precore primers, but not with the core promoter primers, if virus/host junctions occur in that region. The direct repeats, DR1 and DR2, are believed to be hotspots for recombination with host DNA [Nagaya et al., 1987].

With the exception of those amplified from the serum of the HBeAg-positive patient, C23, all sequences had the double mutation at nt 1762 and 1764 (Fig. 3) predicted to down-regulate synthesis of the precore mRNA. In the case of patient C14, more extensive mutations, including deletion of 10 nucleotides (this would cause truncation of the X gene product by 27aa), also were present. There was relatively little heterogeneity in the sequence of this region among cloned products from each patient, compared to that from the precore/proximal core region.

Sequence Analysis of HBV From Asymptomatic Controls

The very high prevalence of core promoter mutations described above might be associated with tumour development or merely reflect the predominant geographic strain of HBV. Therefore, an attempt was made to amplify HBV DNA from the sera of 20 HBsAg-positive individuals who attended an accident and emergency department for reasons not associated with liver disease. Eleven proved to be viraemic, and 3-4 independent clones from each sample were sequenced following PCR amplification (Table II). The double mutation in the core promoter region was found in HBV DNA from three of them (A13, A21, and A23), in one case associated with a precore stop codon (codon 28). A further individual with a precore stop codon had a wild-type promoter sequence.

Notably, no heterogeneity was detected in the HBV nucleotide sequences derived from the control patients,

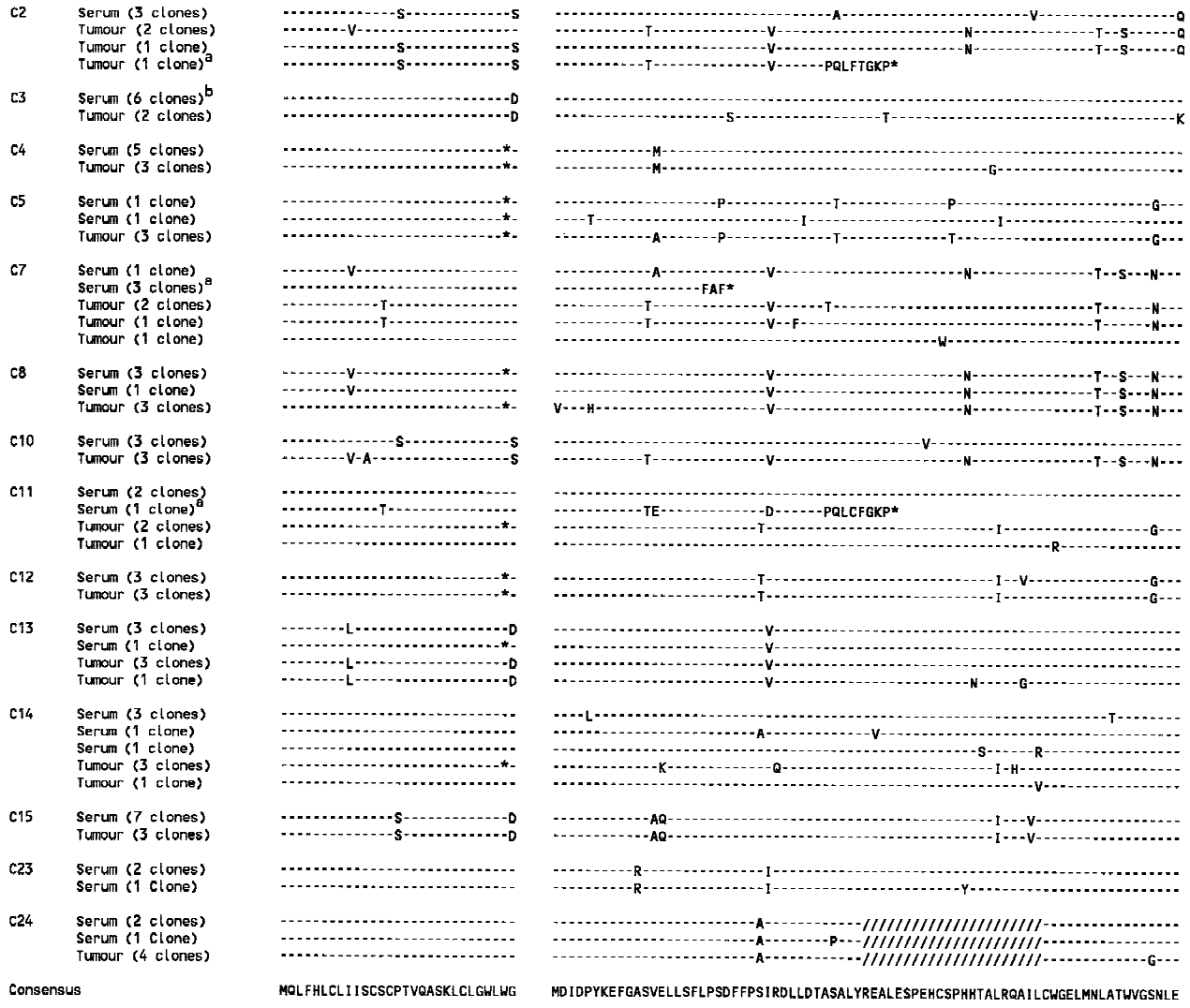

Fig. 1. Amino acid sequences of the precore and distal core region predicted from HBV DNA amplified from serum and tumour. The consensus is shown at the bottom of the figure, and residues that correspond to the consensus are denoted by a hyphen. Deletions are indicated by a series of obliques (//////).*Termination codon.[a] Frameshift caused by single base deletion.[b] One clone encoding Arg at core residue 11 and one clone encoding Pro at core residue 30.

with complete identity of all clones from each individual. However, there was discordance between the HBeAg/anti-HBe status and nucleotide sequence in two cases: patient A24 had a wild-type core promoter sequence and precore codon 28 (in all three clones sequenced) but was anti-HBe positive, and patient A33 was HBeAg positive despite the core promoter double mutation (in 3 clones sequenced). In the latter case, a minor population of wild-type virus, capable of synthesising HBeAg, may have been missed in sequencing only three clones. Alternatively, since the core promoter double mutation does not completely abolish precore mRNA synthesis, detectable levels of HBeAg may have been produced by HBV with the double mutation. HBeAg may have been below detectable levels (or complexed with antibody) in four individuals who tested negative for both antigen and antibody and whose virus had wild-type sequences.

A further patient, A30 (4 clones sequenced), was anti-HBe positive but lacked the double mutation in the core promoter region and a precore stop codon. However, there was a G to A transition, seen also associated with the double mutation in some of the patients with tumour (Table II), and a further, unusual T to G transversion in the promoter region, and it is possible that these affect synthesis of the precore mRNA. Only in one patient, A13, did the precore codon 15 sequence preclude the emergence of a codon 28 stop mutation.

DISCUSSION

The comparison of sequences from currently circulating virus with those integrated in tumour tissue supports the hypothesis that HBV-associated primary liver tumours develop over a period of years following an initial integration event. Figure 2 shows a clear dis-

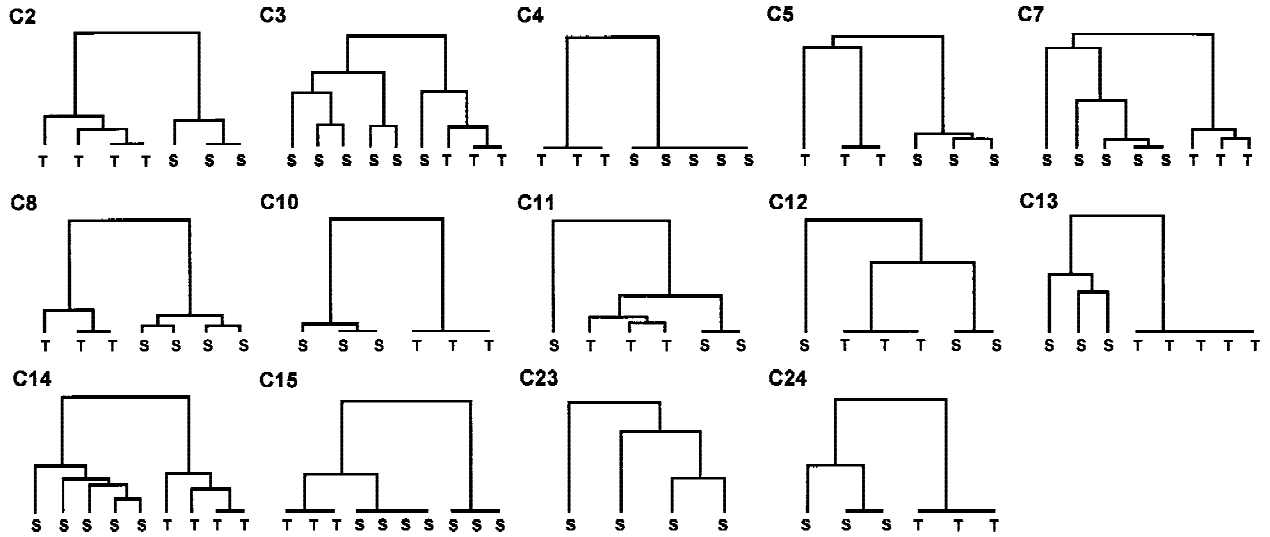

Fig. 2. Dendrograms produced in comparing nucleotide sequences from each individual using PILEUP (Wisconsin Sequence Analysis Package; Genetics Computer Group, Madison, WI, version 9.0). S and T denote sequences derived from serum and tumour, respectively.

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                                ↓ ↓
TGGGGGAGGAGATTAGGTTAAAGGTCTTTGTACTAGGAGGCTGTAGGCATAAATTGGT     wild type
-----------C--------T-A-----------------------------------     case 2, serum (1+2) and tumour (1+2)
----------------------T-A----------G-----------------------     case 3, serum (2+1)
-----------C--------T-A-----------------------------------     case 4, serum (2+10 and tumour (3)
----------------------T-A--------T-----------------------     case 5, (2+1) and tumour (1+2)
----------------A----T-A-T-A----A-----------------------     case 7, serum (1+2)
----------C--------T-A-T-A----A-----------------------     case 7, tumour(2+1)
----------C--------T-A----------G-----------------------     case 8, serum (3) and tumour (2+1)
----------C---A----T-A-T-A----T-----------------------     case 10, serum (1+2)
----------------A----T-A-------TT----------------------     case 11, serum (1+2)
----------------------T-A-----------------------------     case 12, serum (2+2) and tumour (3)
----------------------T-A-----------------------------     case 13, serum (2+1)
----------C--------T-A-----------------------------------     case 13, tumour (2+2)
----------//////////TTAGT-AC---A-----------------------     case 14, serum (2+1) and tumour (2+1)
----------C---A----T-A----------G-----------------------     case 15, serum (1+2)
----------C--------T-A-----------------------------------     case 15, tumour (2)
----------C--------T-A-T------A-----------------------     case 15, tumour (1)
----------------------------------------------------------     case 23, serum (3)
----------C--------T-A-----------------------------------     case 24, serum (2)
----------C--------T-A----------G-----------------------     case 24, tumour (1+2)
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Fig. 3. Analysis of the core promoter region from the 14 tumour patients. The region shown is that in which mutations associated with the HBeAg-negative phenotype may be found (nt 1741 – 1798). A hyphen (-) indicates identity with the wild-type sequence and an oblique (/), a deletion. Numbers in parentheses indicate the number of independent clones sequenced and a plus sign is included where these are derived from separate PCR amplifications. Arrows indicate positions 1762 and 1764.

tinction between these sets of sequences, when compared at the nucleotide level, although many of the changes were silent (Fig. 1). Furthermore, sequences from serum show more diversity than those integrated in tumour DNA, likely reflecting years of error-prone replication by the HBV polymerase. Our anticipation that integration might precede the selection of mutants with a precore stop codon was not borne out; such mutations were rare in our patients and, in the three with precore mutants only, seem to have been present at the time of integration.

It is difficult to interpret missense mutations in the core region as selected by the patient's CTL response. Patients C2, C5, and C12 show the emergence of coding changes in the region codons 48-60, described by Ehata and colleagues [1993], but our sequences do not include the other major hotspot for core amino acid substitutions (codons 84-101). The deletion seen in HBV DNA from patient C24 includes the former region, but since it is present in DNA from the tumour as well as serum, it may have been present in the virus initially infecting this patient, rather than selected by his CTL response. However, we have no evidence that this virus was replication competent because minor species, including the wild type, may have been missed in sequencing a limited number of PCR products.

The most striking outcome of this analysis is the discovery that core promoter mutations are highly prevalent in the HCC patients. Analysis of asymptomatic controls reveals that these HBV mutants do not seem to be as common in asymptomatic carriers of HBsAg in Guangxi. However, it should be noted that anti-HBe was rather less prevalent in these unselected controls (5/11) than in the HCC group (13/14). Elements within

TABLE II. Analysis of Asymptomatic Controls

Patient no. (sex, age)	HBeAg/ anti-HBe	Core promoter	Precore codon 28
Wild type	+/–	GGTTAAAGGT	TGG Trp
A7 (M18)	+/–	GGTTAAAGGT	TGG Trp
A33 (F25)	+/–	GGTTAA**T**G**A**T	TGG Trp
A13 (F60)	–/+	GGTTAA**TGA**T	TGG Trp
A21 (F60)	–/+	GGTTAA**T**G**A**T	**TAG Stop**
A24 (M29)	–/+	GGTTAAAGGT	TGG Trp
A30 (M40)	–/+	G**A**T**G**AAAGGT	TGG Trp
A32 (F22)	–/+	GGTTAAAGGT	**TAG Stop**
A15 (M33)	–/–	GGTTAAAGGT	TGG Trp
A20 (M60)	–/–	GGTTAAAGGT	TGG Trp
A41 (F36)	–/–	GGTTAAAGGT	TGG Trp
A42 (F60)	–/–	GGTTAAAGGT	TGG Trp

the basal core promoter (nt 1744-1851) control the cap sites of the precore and core mRNAs. Point mutations at nucleotides 1762 and 1764, 29 and 27 nt upstream of the precore mRNA cap site (nt 1790 ±1), prevent synthesis of that message and are associated with the HBeAg-negative phenotype [Okamoto et al., 1994]. HBeAg-negative hepatitis B viruses, with codon 28 termination mutations [Kojima et al., 1991; Kosaka et al., 1991], and with core promoter mutations [Sato et al., 1995; Sterneck et al., 1996], have been implicated as causes of acute liver failure.

Baumert et al. [1996] considered the effects of two further mutations in the core promoter, C to T and T to A at positions equivalent to 1766 and 1768 in our Figure 3, in an isolate of HBV from a cluster of cases of fulminant hepatitis B. These mutations were associated with increased transcription of pregenomic RNA, but also with increased encapsidation of RNA beyond that accounted for by the increased transcription. These mutations may be seen in HBV from two of our patients (C7 and C10) and are mimicked in the more extensive rearrangements of HBV DNA from C14. We did not find any mutations in the negative regulatory element (NRE), immediately upstream of the basic core promoter.

Why should these mutations be so common in HBV associated with hepatocellular carcinoma? Increased HBV replication, consequent of increased transcription and packaging of pregenomic RNA, may lead to more of the integration events that precede tumour development in HBV-infected individuals. Accumulation of such pre-neoplastic cells in the liver may increase the chance of further mutational hits, perhaps mediated through the effects of aflatoxin.

Increased replication may be attributed to core promoter mutations if transcription of the pregenomic RNA and expression of the nucleocapsid and polymerase proteins are increased. Increased levels of pregenomic RNA have been reported by some authors but not by others [Buckwold et al., 1996; Raney et al., 1997], the major effect of the mutations being a reduction in precore RNA synthesis. The alterations in transcription patterns probably reflect changes to transcription factor binding sites. Examination of our sequences with the program TFSEARCH suggests that the double mutation may alter the position and orientation of binding sites for HNF4 and COUP-TF and create a binding site for HNF1. Different investigators have observed evidence for a functional role of HNF4 and COUP-TF on the core promoter [Buckwold et al., 1996; Raney et al. 1997] the latter being part of a liver specific complex whose binding is abrogated by the mutations. An additional factor conferring liver specificity to this complex, which does not involve HNF4, has yet to be identified. Overexpression of HNF4 has been reported to increase activity of the core promoter and to repress transcription of the precore transcript [Yu and Mertz, 1996; Raney et al., 1997]. Antagonism between HNF4 and COUP-TF activity is known to occur, so the interaction of these factors in the case of these mutants has yet to be established. The predicted creation of an HNF1 site may be spurious because Gunther et al. [1996] showed that the 1762 and 1764 mutations did not result in HNF1 binding, unlike most other enhancer II mutations. It is possible that these mutations may have a further *cis*-acting effect. The packaging signal ϵ is partially reiterated at the 3′ end of the pregenome and may form extensive secondary structure with nucleotides in the core promoter region [Kidd and Kidd-Ljunggren, 1996]. Furthermore, the core promoter mutations described above are likely to affect any base-pairing in this region. Analysis of these mutations in an in vitro expression system, utilising a greater than genome length HBV template [Ling and Harrison, 1997] may allow dissection of the effects of the mutations, introduced singly into either copy of the repeated region. Recently it has been reported that enhanced replication of these mutants, and more especially of precore mutants, probably is due to a reduction in levels of HBeAg, which has been shown to inhibit virus replication [Scaglioni et al., 1997]. Mutations in the core promoter region also affect the X gene coding sequence. In particular, the double mutation alters codons 130 (lysine to methionine) and 131 (valine to isoleucine) of the X gene. These codons are within regions believed to be important for X gene transactivation function, but the effects of these particular mutations are not known [Kim et al., 1993; Takada and Koike, 1994; Qadri et al., 1995; Renner et al., 1995; Kumar et al., 1996].

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